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Mammalian Models Based on RCAS-TVA Technique

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Abstract: The retroviral vector (RCAS) has been widely used in avian system to study development and diseases, but is not suitable for mammals which do not produce the retrovirus receptor TVA. In this review, we trace the current uses of RCAS-TVA approach in mammalian system with improved strategies, including generation of *tv-a* transgenic mice, use of soluble TVA receptor and retroviral receptor-ligand fusion proteins, improvement of RCAS vectors, and compare a series of mammalian models in variant studies of gene function, development, oncogenesis and gene therapy. All those studies demonstrate that the RCAS-TVA based mammalian models are powerful tools for understanding the mechanisms and target treating of human diseases.

Key words: RCAS vector; TVA; Specific promoter; Transgenic animal; Mammalian model

基于 RCAS-TVA 技术的哺乳动物模型

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摘要：近年来，鸟类逆转录病毒载体（RCAS）及其受体（TVA）系统在哺乳动物转基因模型中得到广泛应用。本文对转 *tv-a* 基因小鼠的制备、特异性启动子选择、RCAS 载体的改进等方面进行综述，展示近来 RCAS-TVA 系统在哺乳动物所取得的成果，并对 RCAS-TVA 基因转移技术的应用前景作一展望。

关键词：RCAS 载体；TVA；特异性启动子；转基因动物；哺乳动物模型
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Retroviruses are enveloped viruses possessing a RNA genome, and replicate *via* a DNA intermediate. They rely on the enzyme, reverse transcriptase, to perform the reverse transcription of their genomes from RNA into DNA, which can then be integrated into the host's genome. The viruses then replicate as part of the cell's DNA (http://en.wikipedia.org/wiki/Retrovirus). So they have the ability to introduce new genetic information into the chromosomes of target cells, and serve as vehicles for transfer of exogenes (Orsulic, 2002). To date, retroviral vectors have been wildly developed to study gene function and therapy, developmental processes, oncogenesis, and so forth (Logan & Tabin, 1998; Hu & Pathak, 2000; Barton & Medzhitov, 2002; Kawakami et al, 2003; Pao et al, 2003; Harpavat & Cepko, 2006; Du & Li, 2007). Among those retroviruses, the avian sarcoma-leukosis virus-A (ASLV-A)-derived vector called RCAS (Replication Competent ASLV long terminal repeat with Splice acceptor) is used most extensively in avian system, because high titer viral stocks can be harvested in avian cells without helper components. RCAS vectors are derived from the SR-A strain of Rous sarcoma virus (RSV) by deleting the *src* oncogene with a multi-cloning site where exogenes can

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be inserted (Hughes & Kosik, 1984; Hughes et al, 1987; Petropoulos & Hughes, 1991; Boerkoel et al, 1993). The multi-cloning site can stably accommodate inserts up to 2.5kb (Fig. 1). Expression of the inserted genes can be driven by either the viral long terminal repeat (LTR) or an appropriate internal promoter (Petropoulos et al, 1992; Du et al, 2006). A loss-of-function method, RCAS-RNAi (RNA interference) technique, has been verified to be efficient in "knocking down" the specific genes in avian developing craniofacial tissues, the limb bud, dorsal root ganglion, and the retina (Kawakami et al, 2003; Pekarik et al, 2003; Harpavat & Cepko, 2006).

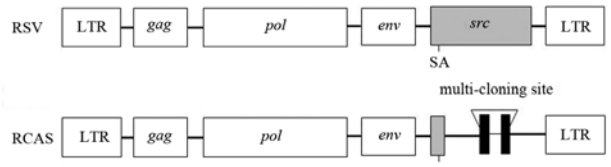

Fig. 1 Constructs of the RSV and RCAS vector

The diagrams show the organization of viral DNA genomes and the location of genes (*gag*, *pol*, *env*, and *src*) and long terminal repeat (LTR). The *src* oncogene of RSV carries a splice acceptor (SA), which is retained in RCAS. The *src* oncogene has been deleted and replaced by a multi-cloning site in RCAS vector.

However, RCAS vectors can not be used in mammalian system directly without any improvement on the mammalian cells, which do not express the surface receptors for virus entry and infection. TVA, a member of the low-density-lipoprotein receptor family, is encoded by the *tv-a* gene and acts as the receptor for ASLV-A in avian cells (Bates et al, 1993; Young et al, 1993). mRNA transcribed from the *tv-a* gene is alternatively spliced to produce at least two proteins, a transmembrane and a GPI-anchored isoform (Bates et al, 1993). The mammalian cells are able to be infected and allow for genome integration by ASLV-A or RCAS virus if the cells are engineered to express TVA ectopically on the surface, and both of the isoforms are sufficient to permit infection of mammalian cells (Bates et al, 1993; Young et al, 1993).

Compared with the general mammalian counterparts, RCAS vectors can be constructed to encode all of the proteins required for assembly of infectious particles in addition to the transferred gene of interest, so they do not require helper cells (Hughes et al, 1987; Petropoulos & Hughes, 1991; Boerkoel et al, 1993). High-titer viral stocks can be produced in avian cells (Himly et al, 1998; Schaefer-Klein et al, 1998). Viral proteins are inefficiently produced in mammalian cells, so the vectors can not spread from the target animals and cell-to-cell spreading within any individual is also prevented (Wills et al, 1989; Berberich et al, 1990). The lack of viral proteins also decreases the immune response by the host (Pinto et al, 2000). Furthermore, the most specific advantage of RCAS vectors is that multiple genes can be transferred sequentially into the target cells of a single transgenic animal (Federspiel et al, 1994; Holland et al, 1998; Murphy & Leavitt, 1999). This feature should be attributed to the sufficient supplement of TVA receptor which is not blocked by the poorly expressed viral envelope protein in mammalian cells. Recent experiments indicate that the RCAS vectors have the ability to infect non-dividing mammalian cells, including the primary neurons, although there is no direct evidence (Hatziioannou & Goff, 2001; Katz et al, 2002; Greger et al, 2004). The procedure to generate a RCAS-TVA based model is shown in Fig. 2.

Some potential limitations for using the RCAS-TVA system, however, should be given close attention. Target cells, tissues and organs must express the receptor TVA. Therefore, it is crucial to generate TVA transgenic animals before RCAS infection. RCAS can only accommodate an insert of less than 3 kb, but this limitation can be partially overcome by using pseudotyped vectors. The MLV (moloney murine leukemia virus), carrying capacity of insert up to 6-7kb, has been efficiently pseudotyped with ASLV envelope protein (Soneoka et al, 1995; Murphy & Leavitt, 1999). But, integration site of viral DNA can not be controlled. Efficiency of infection is dependent on the accessibility of the organ and the proliferation rate of target cells. Description of various advantages and limitations of using the RCAS-TVA system has been reviewed in detail by Orsulic (2002).

1 Transgenic mammals expressing TVA molecules

1.1 Choice of specific promoters

The key element to produce *tv-a* transgenic animals is the tissue-specific and the lineage-specific promoters which decide specific expression of TVA in target cells, tissues and organs. Therefore, a nucleotide fragment

consisting of the *tv-a* cDNA and a proper promoter must be constructed before RCAS infection. The specific promoters currently used to drive expression of *tv-a* gene in mammalian system are summarized in Tab. 1.

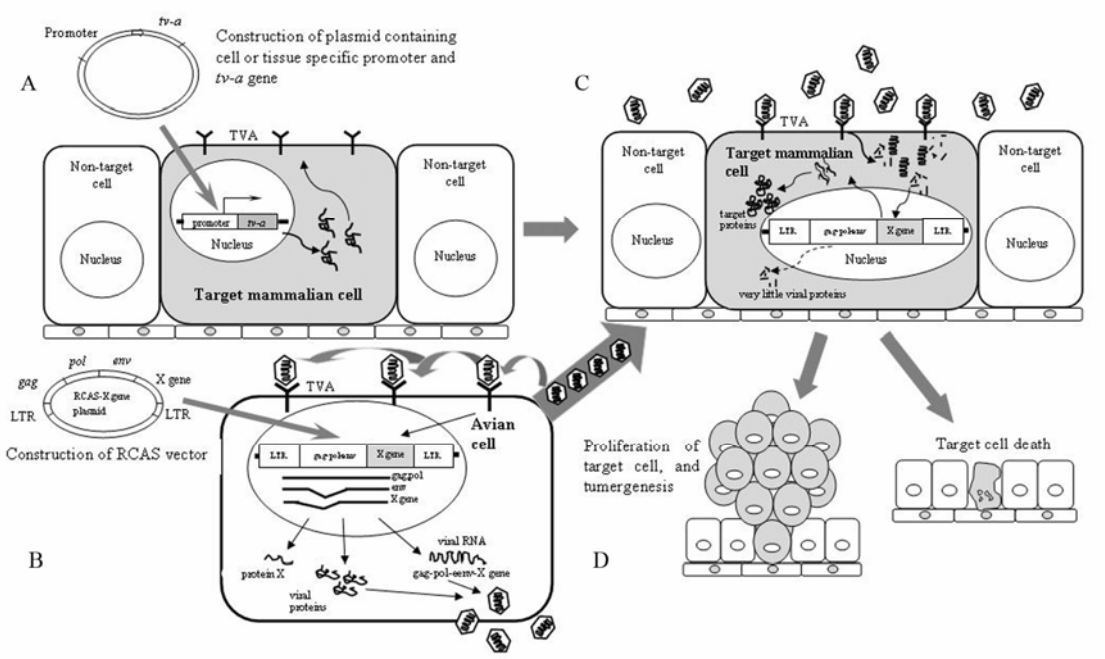

Fig. 2 Schematic drawing of the RCAS-TVA technique in mammalian system

A: The target mammalian somatic cells are engineered to express TVA receptor under a tissue-specific promoter and therefore are susceptible to virus infection. B: Avian cells are transfected with a plasmid encoding the replication-competent, avian viral vector RCAS which contains the viral genes, *gag*, *pol* and *env* and a gene of interest (X gene). The viruses are produced in high titer and can infect avian cells again through the TVA receptors on the surface of cells. C: The mammalian cells expressing TVA are infected by RCAS vectors, and only the protein encoded by X gene is efficiently produced. Because very little viral proteins are produced, no infectious RCAS are replicated in mammalian cells. Therefore, the TVA receptors can be used repeatedly with different vectors. The neighbor cells can not be infected by RCAS because of their deficiency of TVA receptor. D: The target cells transfected with the genes of interest will show different destinies, such as proliferating or dying.

Tab. 1 Summary of the target cells, tissues and organs expressing TVA and promoters used to drive expression of *tv-a* in mammalian system

Target	Promoter/contex	Reference
Bone	BSP	Li et al, 2005
Brain	GFAP	Holland & Varmus, 1998; Yamashita et al, 2006
	nestin	Holland et al, 1998
Hematopoietic cell	GP-Ibα	Murphy & Leavitt, 1999
Liver	albumin	Lewis et al, 2005
Lung	SPC	Fisher et al, 1999
Mammary epithelial cell culture	MACT	Phillips et al, 2006
Mammary gland	MMTV	Du et al, 2006
MEFs	β-actin	Pao et al, 2003
Most or all tissues	β-actin	Federspiel et al, 1996
Neural crest cell	TRP2	Fisher et al., 1999
Ovary	β-actin , keratin	Orsulic et al, 2002; Xing & Orsulic, 2005
Pancreas	elastase I	Kruse et al, 1993; Lewis et al, 2003
RK3E cell line	CMV	Fu et al, 2005
Skeletal muscle and heart	α-actin	Federspiel et al, 1994
Vascular endothelium	Tie2	Montaner et al, 2003

BSP, bone sialoprotein; CMV, cytomegalovirus; GFAP, glial fibrillary acidic protein; GP, glycoprotein; MACT, mouse β-actin; MEFs, murine embryonic fibroblasts; MMTV, mouse mammary tumor virus; RK3E, rat kidney epithelial cell line; SPC, surfactant protein-C; TRP2, tyrosinase-related protein-2.

Additionally, numerous studies have indicated that some other promoters, including the ovary specific promoter (OSP1) and the high-affinity folate receptors promoter (HAFR) (Godwin et al, 1995; Goldsmith et al,

1999), the modified rat probasin (rPB) promoters (Furuhata et al, 2003), and the neuroactive peptide cholecystokinin (CCK) promoter (Chhatwal et al, 2007), are of potential value for tissue specific expression of the *tv-a* gene.

1.2 Both isoforms of TVA molecule are sufficient for acceptance of the RCAS vectors

As mentioned earlier, two isoforms, a transmembrane and a GPI-linked one, have been identified. In the current *tv-a* transgenic mice, the GPI-anchored isoform is commonly used (Federspiel et al, 1996; Holland et al, 1998; Du et al, 2006), but the transmembrane isoform has also be used successfully in the study of Murphy & Leavitt (1999). Therefore, both isoforms can accept the RCAS vectors although the physiological functions have not been determined.

1.3 Gene transfer methods

The most widely used method to generate transgenic animals is to microinject foreign DNA into the pronucleus of a fertilized egg. Pronuclear microinjection is conceptually straightforward, although it demands special equipment and technical skill, and has the additional feature that any cloned DNA can be used (Palmiter & Brinster, 1986). The primary mammalian model based on the RCAS-TVA approach was developed in transgenic mice expressing TVA specifically in muscle cells. They were generated by microinjecting a nucleotide fragment consisting of the *tv-a* cDNA and chicken α-actin promoter fragment into fertilized mice eggs (Federspiel et al, 1994). Subsequently, Holland et al. generated mice expressing TVA on the surface of glial cells by microinjection of *Gtv-a* transgene, which is a 2.2 kb fragment of the GFAP promoter driving expression of the quail *tv-a* cDNA and a fragment from the mouse protamine gene (*MP-1*) supplying an intron and signal for polyadenylation (Holland et al, 1998, 2000). Using the microinjection method, then, *tv-a* transgenic mice were extensively created to accept RCAS vectors in cancer models of mammary, ovary, pancreas, liver, lung, brain, vascular endothelium, melanoma, and other cell types (Korfhagen et al, 1990; Holland & Varmus, 1998; Holland et al, 1998; Fisher et al, 1999; Orsulic et al,2002; Montaner et al, 2003; Lewis et al, 2003, 2005; Pao et al, 2003; Fu et al, 2005; Du et al, 2006) and development models, including neuron, hemapoietic cell lines, and other organs (Doetsch et al, 1999; Murphy & Leavitt, 1999; Fisher et al, 1999).

However, the microinjection method has some potential limitations: 1. limited success in producing transgenic animals of larger species; 2. requirement of special equipment for DNA microinjection and high technical skills; 3. labor intensive. The new mean has been developed by using sperm cells, including spermatogonia, as the vehicle to deliver exogenous DNA into oocytes, and is therefore called "sperm-mediated gene transfer" (SMGT, Lavitrano et al, 1989). Based on SMGT, transgenic rats (Hamra et al, 2002; Orwig et al, 2002), pigs (Honaramooz et al, 2002) and goats (Honaramooz et al, 2003) have now been produced. As the improved method of SMGT, testis-mediated gene transfer (TMGT) has been demonstrated to be practical in delivering foreign DNA directly into the interstitial space of adult mammalian testes (Fig. 3A), and then the exogenous DNA is transmitted to oocytes *via* fertilization (Sato et al, 1999; Sato & Nakamura, 2004). Recently, He et al (2006) further indicated that transgenic efficiency of TMGT was very high in both F1 and F2 mice offspring (41% and 37% respectively), and that TMGT was suitable for creating transgenic animals. The TMGT technique is very simple and convenient. A needle, a plastic disposal syringe, and a dissecting microscope are sufficient for delivery of DNA. The TMGT technique opens a new perspective for generating *tv-a* transgenic mammals (Fig. 3B), although it requires further improvement.

More recently, Yang et al (2007) established a rapid procedure for obtaining transgenic mice by directly injecting exogenes into the ovaries of fertile mice, called ovary mediated gene transfer (OMGT). After natural fertilization, healthy transgenic mice were obtained, and the introduced foreign gene was inherited by F1 offspring (64.9%) and transmitted to F2 progeny (66.94%) stably. The foreign gene was found to be not only integrated into the genome with a high frequency of 85.71% (multiple site versus single site insertions analyzed by FISH), but also translated into a functional protein and transferred to the next generation. Although the procedure is somewhat more complicated than TMGT, OMGT is still a useful technique with a much higher success rate for creating transgenic mammals *via* efficient and functional integration of the foreign gene into the host genome and stable transmission of the

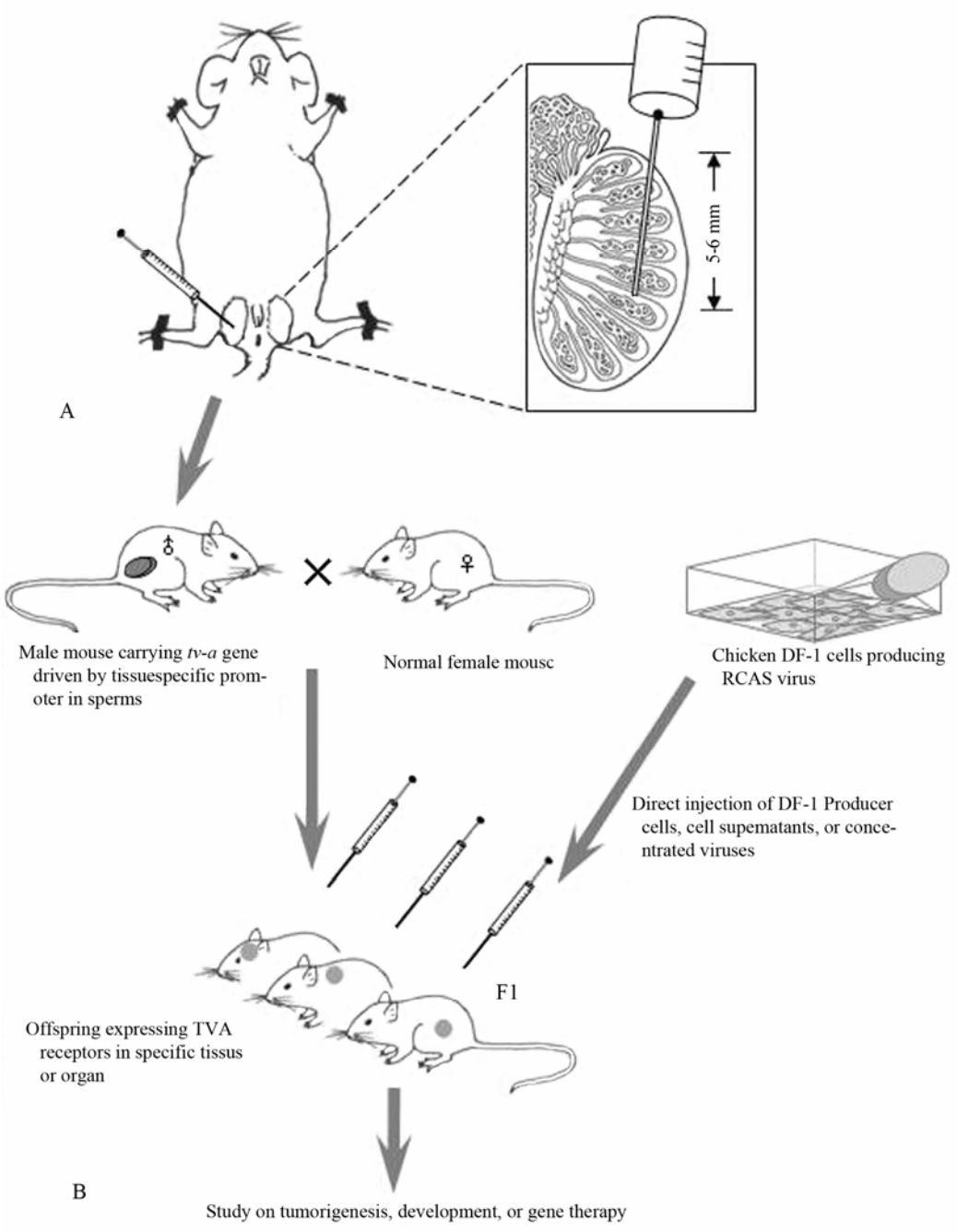

Fig. 3 Schematic drawing of testis-mediated gene transfer (TMGT) method and retroviral gene delivery to mammals *in vivo*
A: Injection of solution containing *tv-a* gene driven by tissue-specific promoter and liposomes (in some cases) is performed at the corner of the testis near the caput epididymis to a depth of 5-6mm (Sato et al, 1999). B: The adult male mice carrying *tv-a* gene driven by a tissue-specific promoter are mated with normal females，and the promoter-*tv-a* sequence will be delivered into zygotes by sperms. The offspring (F1 generations) are examined, and only those expressing TVA in specific tissues or organs are left for retroviral infection. Chicken DF-1 cells transfected with RCAS vectors carrying genes of interest are propagated to obtain high titer viruses. Producer DF-1 cells, cell supernatants, or concentrated viruses can be used to infect TVA-expressing mice. Tissue-specific infection can be achieved by direct injection of viruses or virus-producing cells into an organ in which TVA is expressed. The anatomy atlas of testis is modified from the website http://msjensen.cehd.umn.edu/Webanatomy/image_database/Reproductive/testis2.gif.

foreign gene to the offspring. Simplicity of procedure and cost-effectiveness are the advantages of OMGT used for *tv-a* transgenic animals in contrast to other traditional methods, such as pronuclear microinjection.

2 RCAS vectors used for mammalian system

2.1 Modified vectors for overcoming the limited insertion size

As mentioned earlier, RCAS can only accommodate an insert of less than 3 kb. This may not be a significant problem because most cDNAs studied are less than 2.5 kb (Fisher et al, 1999). This limitation can, however, be overcome by using RCAS and ASLV-A Env pseudotyped HIV and MLV vectors, respectively (Murphy & Leavitt, 1999; Lewis et al, 2001), even if more than 3 kb sequences must be inserted.

2.2 Improved vectors for infection of a broad range of cell types

Although some reports have shown that RCAS can infect non-dividing cells (Lu et al, 1999; Hatziioannou & Goff, 2001), low efficiency suggests entrance of viral DNA into nucleus depends on mitosis of host cells. To overcome this limitation, Lewis et al (2001) succeeded in using a pseudotyped replication-deficient HIV-1 based lentiviral vector to infect non-dividing TVA positive cells. However, there is no evidence that can clarify the efficiency of this vector *in vivo*. Therefore, further studies are required to improve the ability of RCAS vectors into the genome of non-dividing host cells.

2.3 Vectors used in mammalian system

The RCAS family consists of a group of vectors for variant demand. Actually, the current vectors used in mammalian system, have an *env* gene from a murine retrovirus instead of one from the ASLV. These vectors are named as RCASBP, in which *env* gene is derived from an amphotropic virus or ecotropic virus. The properties of RCASBP vectors are summarized on the website:
http://home.ncifcrf.gov/hivdrp/RCAS/tables.html#table2.

3 Overview of current mammalian models based on RCAS-TVA technique

The primary mammalian model based on RCAS-TVA approach was developed in mice (*Mus musculus*) by Federspiel et al (1994). This work opens a new way to study development and oncogenesis, and sheds light on models for tissue-specific gene therapy.

3.1 Use of RCAS-TVA based models to study developmental processes

RCAS-TVA based method has been proven to be useful in developmental studies in mammalian system. Murphy & Leavitt (1999) used the *GP-Ibα* regulatory sequences to achieve megakaryocyte-lineage of mice restricted expression of TVA. They infected the cells with RCAS-*PURO* (expresses puromycin-resistance gene) and RCAS-*AP* (expresses human placental alkaline phosphatase) *in vitro* and *in vivo*, then generated and characterized a pure population of primary CD41-positive megakaryocyte progenitors. The *in vitro* study indicated that IL-3 inhibits the development of mature megakaryocytes. Doetsch et al (1999) infected SVZ (subventricular zone) astrocytes of *tv-a* transgenic mice with RCAS-*AP in vivo*, and the AP-positive cells were examined and traced. They demonstrated that SVZ astrocytes act as neural stem cells in normal brain. Study of lung development has been reported using the RCAS-TVA model (Fisher et al, 1999). Lung buds of the SPC-*tv-a* transgenic mice were infected with different RCAS viruses to study the effects on branching morphology *in vitro*. To study bone development *in vivo*, Li et al (2005) established the BSP-*tv-a* transgenic mice which selectively expressed TVA in skeletal tissues. After infecting with RCASBP-*Cbfa1/Runx2*, bone and tooth formation was delayed. They validated this model as a unique system for studying molecular events associated with bone formation *in vivo*.

Dunn et al (2000, 2001)have infected the neural precursor cells and the melanoblasts expressing TVA driven by nestin and Dopachrome tautomerase promoter (DCT) with RCAS-*Wnt*, RCAS-*lacZ* (β-galactosidase) and RCAS-*Tyr* (tyrosinase) respectively in primary culture and *in utero*. They demonstrated that the RCAS-TVA method was useful to study the development of neural systems. Recently, the RCAS-TVA system was successfully adapted by Yamashita and colleagues to study neurogenesis *in vivo* (Yamashita et al, 2006). They traced maturation of neurons by infecting the GFAP *tv-a* (*Gtv-a*) transgenic mice with RCAS-*EGFP* (CAG-CAT-enhanced green fluorescent protein), and indicated that SVZ-derived neuroblasts differentiated into mature neurons in the post-stroke striatum.

3.2 Use of RCAS-TVA based models to study oncogenesis

Currently, most models of tumors are traditionally germ-line models constructed by transgenic or knockout approaches. The major limitation of these models is that the initiation and progression of carcinogenesis can not be understood. However, the RCAS-TVA method overcomes this limitation and allows investigation of the carcinogenic potential of candidate oncogenes in somatic

cells *in vivo* without creating individual transgenic lines (Du & Li, 2007). To date, several oncogenes have been studied in murine system using this technique. The variant cancers or tumors and oncogenes studied are summarized in Tab. 2.

Cancer is thought to be associated with multiple genetic alterations. Microarray analysis of ovarian cancer has demonstrated that oncogenesis of ovarian neoplasms is controlled by many genes, and that changes in expression of these genes correlate with malignancy potential (Warrenfeltz et al, 2004). To study the effect of multiple genes on carcinogenesis, the RCAS-TVA system provides a flexible method to deliver several genes simultaneously or sequentially. Holland et al (2000) infected the *Ntv-a* transgenic mice with a combination of DF-1 cells infected with and producing RCAS-*Ras* and RCAS-*Akt*. They found that combination of activated Ras and Akt induces high grade gliomas with the histological features of human glioblastoma multiformes (GBMs) although neither activated Ras nor Akt alone is sufficient to induce GBM formation.

Orsulic et al (2002) isolated ovarian cells from TVA transgenic mice deficient for *p53*, and infected the target cells with RCAS-*Myc*, RCAS-*Ras*, and RCAS-*Akt*. Their study showed that addition of any two of the oncogenes *Myc*, *Ras*, and *Akt* were sufficient to induce ovarian tumor formation when infected cells were injected into the recipient mice at subcutaneous, intraperitoneal, or ovarian sites. They demonstrated that the ovarian surface epithelium is the precursor tissue for these ovarian carcinomas, and that introduction of oncogenes causes phenotypic changes in the ovarian surface epithelial cells.

A mouse model for hepatocellular carcinoma was generated by infecting *tv-a* transgenic wild-type and *p53* null mice with RCAS-*PyMT* (Lewis et al, 2005). Tumors were induced in both wild-type and *p53* null mice, but only in the mice lacking an intact *p53* gene the resulting tumors were poorly differentiated, invasive, and metastatic to the lungs. This study demonstrates that metastasis is dependent on both the oncogene and the absence of *p53*.

3.3 Use of RCAS-TVA based models to study gene function

The lost-of-function and "knock out" techniques are robust and practical for studying gene function in mammalian system. The retroviral vectors have been validated to express short hairpin RNA (shRNA) under the control of an RNA polymerase III promoter for the purpose of inhibiting gene expression in a sequence-specific manner (Brummelkamp et al, 2002; Hemann et al, 2003; Rubinson et al, 2003).

To date, RNA interference (RNAi) technique has been incorporated successfully with the RCAS-TVA method to study gene function in avian development. Bron et al (2004) knocked the neuropilin-1 (Nrp-1) receptor in chick embryos using the RCAS-RNAi technique. They found that Sema3A-induced growth cone was inhibited in dorsal root ganglion (DRG) neurons. This result demonstrated the functional knockdown of Nrp-1. Harpava and Cepko (2006) delivered hairpins mediating RNA interference to the

Tab. 2 Cancers/tumors and oncogenes studied in murine system by RCAS-TVA approach

Cancer/tumor	Oncogene	Target	Reference
Breast cancer	*Cre*	Mammary gland *in vivo*	Fisher et al, 1999
	PyMT, Neu		Du et al, 2006
Liver cancer	*PyMT, Myc*	Liver parenchyma *in vivo*	Lewis et al, 2005
Nervous system tumors			
Astrocytoma	*Ras, Akt*	Brain *in vivo*	Holland & Varmus, 1998
Glioblastoma			Holland et al, 2000
Oligoastrocytoma	*PDGF-B*	Brain *in vivo*, Primary brain cell cultures	Holland & Varmus, 1998; Dai et al, 2001
Oligodendroglioma			Dai et al, 2001
Primitive neuroectodermal tumors (PNETs)	*Myc*	Neural progenitor cells, brain *in vivo*	Fults et al, 2002
Ovarian cancer	*Myc, Ras, Akt*	Ovarian cells in culture	Orsulic et al, 2002
		Ovarian cancer cell lines and tumors with defined genetic alterations	Xing & Orsulic, 2005
Pancreatic cancer	*PyMT, Myc*	Pancreas *in vivo*	Lewis et al, 2003

PDGF-B, platelet derived growth factor-b chain.

developing chick eye by RCAS viruses. They 'knocked down' specific genes in infected areas of the retina. The knock down persisted as the retina matured and could be detected using *in situ* hybridization. Furthermore, the amount of retinal tissue affected could be controlled by manipulating the degree of infection.

In mammalian system, Bromberg-White et al (2004) created a RCAS vector capable of expressing shRNA that inhibits the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene, and reduces GAPDH expression in cell line A375. They demonstrated that RCAS vectors can be used to stably express shRNA to inhibit gene expression in loss-of-function analyses of specific genes *in vitro* as well as *in vivo*.

The RCASBP-Y vector has been modified to incorporate "Gateway" site-specific recombination cloning of genes into the viral construct, and will allow for the efficient transfer and expression of cDNAs required for functional genomic analyses in both avian and mammalian model systems (Loftus et al, 2001).

3.4 Use of soluble TVA receptor, TVA-ligand bridge proteins and RCAS system for gene therapy

RCAS system has been considered useful for gene therapy of cancers (Orsulic, 2002; Xing & Orsulic, 2005). However, gene therapy is dependant on the ability of target cancer cells to accept the viral vectors carrying therapeautic or suicide genes. Many TVA receptor transgenic models have been generated in mammalian system to accept RCAS vectors, simultaneously new methods are developed. Several studies have indicated that the membrane TVA receptor is not an absolute requirement for virus infection, and RCAS vectors linked with a soluble TVA can be delivered into receptor-deficient cells (Snitkovsky & Young, 1998; Damico & Bates, 2000; Contreras-Alcantara et al, 2006). The viral receptor function of TVA is determined by a 40-residue, cysteine-rich motif called the LDL-A module, which is highly homologous to the human low-density lipoprotein receptor (LDLR) ligand-binding repeats (LDL-A modules). It has been demonstrated that the LDL-A module of TVA is necessary and sufficient to mediate efficient EnvA binding and ASLV-A infection (Rong & Bates, 1995). Therefore, the soluble TVA receptor is an ideal candidate for transferring RCAS to target cells deficient in membrane TVA.

Additionally, several proteins consisting of a TVA receptor-ligand fusion structure have been developed to serve as bifunctional bridge to surface receptors of target cells and RCAS vectors (Fig. 4). The bridge proteins contain the extracellular domain of TVA and a peptide which can bind to surface receptors of target cells (Orsulic, 2002). Several authors have succeeded in infecting the mammalian cells expressing cognate cellular receptors using the bridge proteins consisting of the domain of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), or heregulin (Boerger et al, 1999; Snitkovsky et al, 2000, 2001; Snitkovsky & Young, 1998, 2002). This method provides a flexible way to target entry of RCAS vectors to mammalian cells.

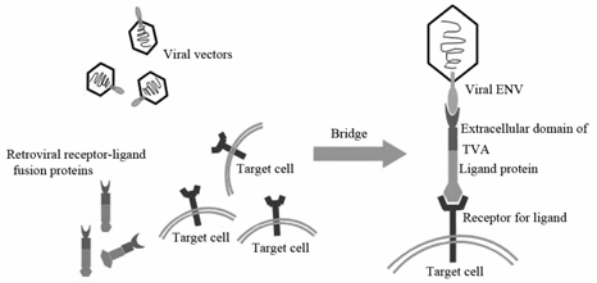

Fig. 4 Delivery of RCAS vectors into target mammalian cells by retroviral receptor-ligand fusion proteins

The fusion protein is comprised of the extracellular domain of TVA fused with a ligand protein which permits it to bind to RCAS viral ENV and to cell surface receptor respectively.

Hu et al (2007) investigated for the first time the characteristics of RCAS as an alternative vector system for transduction of hematopoietic stem and progenitor cells. The new vectors were modified by replacing the avian *env* gene with the gene encoding amphotropic or ecotropic murine Env protein, which allows RCAS vectors to infect mammalian cells efficiently (Barsov & Hughes, 1996; Barsov et al, 2001). They used nonhuman primate autologous transplantation models to test whether the RCAS vectors can efficiently transduce rhesus macaque $CD34^+$ hematopoietic stem and progenitor cells. This study showed that RCAS vectors could efficiently and stably transduce the $CD34^+$ hematopoietic progenitor cells with an efficiency of transduction of up to 34% *in vitro*, and that highly polyclonal hematopoietic reconstitution in myeloid and lymphoid lineages was observed up to 18 months post-transplantation in animals transplanted with RCAS vector-transduced autologous $CD34^+$ cells. Hu et al

(2007) indicated that the RCAS system should be explored and further optimized for gene therapy applications targeting hematopoietic stem and progenitor cells.

4 Conclusion and future research

This review shows that the RCAS-TVA based technique is very useful and valid in various areas, including basic biology, medicine and clinical research. To date, a large number of RCAS vectors (and ancillary tools, including soluble receptors, receptor-ligand fusion proteins, mammalian cell lines expressing receptors, and *tv-a* transgenic mice) have been developed for mammalian system, and the number of mammalian models (especially the mouse models) is constantly increasing. These models can be used for a number of different study purposes: gene function, development, carcinogenesis and gene therapy.

Bioreactor is also a novel potential for the use of RCAS-TVA system in the future. Pronuclear microinjection is the major method today for the production of transgenic animal bioreactor, but repeated operation is inevitable to create different transgenic animals producing different bioactive proteins. The RCAS-TVA technique provides a convenient and flexible way to produce a variety of biological products in one transgenic animal, in which different target genes can be introduced to the tissues or organs expressing TVA molecules simultaneously, sequentially or repeatedly. For the objective of producing pharmaceuticals for treating human diseases, mammary glands may be the ideal tissue for use of the RCAS-TVA system.

The TMGT and OMGT techniques open a new perspective in creating *tv-a* transgenic mammals. Compared with the conventional pronuclear microinjection approach, use of TMGT and OMGT is much cheaper and faster with quick and effective delivery of genes to target tissues (Sato, 2005; Yang et al, 2007). Therefore, the cost for the whole experiment is decreased. The current studies demonstrate that gene knock down can be made *in vitro* and *in vivo* by using the RCAS system expressing RNAi. It can be predicted that the RCAS-RNAi technique will be very useful for gene function (or loss-of-function) studies. To improve the infectious ability of RCAS vectors to cells absent of mitosis, more and more vectors are being developed. Furthermore, novel uses of the RCAS-TVA system including infection of non-dividing somatic cells, and neurons, are being developed in mammalian models.

On the whole, the RCAS-TVA based mammalian model is a powerful tool for understanding the mechanism and target treatment of human diseases. We anticipate that new uses for the RCAS-TVA method in mammals will be developed with a better understanding of retroviruses, its hosts, and using other new biological techniques.

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